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CCAAT/enhancer binding protein β binds to and activates the P_{670} promoter of human papillomavirus type 16

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Abstract

The P_{670} promoter of HPV16 directs transcription of the virus late genes in the differentiating epithelium. We found that CCAAT/enhancer binding protein β (C/EBPβ), a key transcription factor that induces the terminal differentiation of keratinocytes, enhanced the P_{670}-driven transcription in transient reporter assays in HeLa cells and human primary keratinocytes, whereas it inhibited, as reported previously, the transcription from the early P_{97} promoter. An electrophoretic mobility shift analysis identified two binding sites in the upstream region of P_{670} for a bacterially expressed C/EBPβ. A chromatin immunoprecipitation analysis demonstrated that C/EBPβ bound to these sites of the P_{670} reporter plasmid in HeLa cells. Nucleotide substitutions in these sites in the reporter plasmid abrogated the enhancement by C/EBPβ in the transient HeLa and keratinocyte assays, indicating that the C/EBPβ-binding to these sites is required for the enhancement of transcription from P_{670}. These results suggest that C/EBPβ is involved in enhancing transcription from the P_{670} during keratinocyte differentiation.

Keywords: HPV16; P_{670}; C/EBPβ; Keratinocyte differentiation

Introduction

Human papillomaviruses (HPVs), small icosahedral viruses with circular double-stranded DNA genomes of 8 k base pairs (bp), cause proliferative lesions of the skin or mucosa (zur Hausen, 1996). To date more than 100 genotypes of HPVs have been identified and classified based on the homology of genomic DNA. HPVs that infect the genital epithelia are divided into two groups: low-risk types such as HPV type 6 (HPV6) and HPV11 found mainly in benign condyloma and high-risk types such as HPV16, HPV18, HPV31, and HPV33 found in cervical cancer (Longworth and Laimins, 2004; zur Hausen, 2000).

All HPVs have an overall similarity in the genomic organization: the early genes encoding viral nonstructural proteins (E1, E2, E4, E5, E6, and E7 proteins), the late genes encoding two capsid proteins (L1 and L2 proteins), and the non-coding long control region (LCR) (between the L1 and E6 genes) carrying the replication origin (Fehrmann and Laimins, 2003). Most of the early genes are transcribed from the promoter in LCR (such as HPV16 P_{97} and HPV31 P_{97}), and the E1 and late genes are transcribed from the promoter that is located within E7 gene (such as HPV16 P_{670} and HPV31 P_{742}) (Grassmann et al., 1996; Hummel et al., 1992).

The life cycle of HPVs is closely associated with epithelial differentiation (Longworth and Laimins, 2004). HPVs reach and infect the basal cells of the stratified epithelia through small epithelial lesions. In the basal cells, the viral DNA is maintained as episomes, and the early genes are transcribed at a very low level. When the host cells initiate terminal differentiation, the HPV DNA starts to replicate and to be transcribed efficiently. Then, HPV virions are generated in the upper layers of the epidermis or mucosa and released from them.

Transcriptional activities of the HPV promoters are drastically changed during the terminal differentiation of the host keratinocytes. Studies using immortalized human keratinocytes that harbor HPV16 or HPV31 DNA have demonstrated that HPV16 P_{97} or HPV31 P_{97} is active in undifferentiated cells, while HPV16 P_{670} or HPV31 P_{742} is suppressed (Grassmann et al., 1996; Hummel et al., 1992). Differentiation of the host cells induces a massive increase in transcripts from P_{670} or P_{742}, leading to expression of the L1 and L2 capsid proteins and E1 replication protein (Klumpp and Laimins, 1999; Ozbun and

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Meyers, 1997; Ruesch et al., 1998). Although the viral DNA replication coincides with the activation of P_{670} or P_{742}, it is not a prerequisite for the activation (Bodily and Meyers, 2005; Spink and Laimins, 2005). Cellular proteins in the differentiating epithelium are expected to directly regulate the transcriptional activities of the promoters, yet the molecular mechanisms of the activation and suppression are largely unknown.

Epidermal differentiation is regulated through the action of cellular transcription factors, which include AP-1 family proteins (Eckert et al., 1997), POU-domain proteins (Ryan and Rosenfeld, 1997), NF-kB family proteins (Seitz et al., 1998), and CCAAT/enhancer binding proteins (C/EBPs) (Maytin et al., 1999; Zhu et al., 1999). The C/EBP family of transcription factors are known to play a role in a wide range of biological processes, such as inflammation, the control of energy metabolism, and cellular proliferation and differentiation (Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002).

The C/EBP family is composed of six members: C/EBPα, -β, -δ, -γ, -ε, and -ζ (Ramji and Foka, 2002). In keratinocytes, C/EBPα, -β, -δ, and -ζ are expressed, and their expression levels fluctuate during the differentiation (Maytin and Habener, 1998; Smith et al., 2004). Of particular interest is that overexpression of C/EBPβ in keratinocytes causes growth arrest and the induction of early differentiation markers (Zhu et al., 1999), suggesting a crucial role of C/EBPβ in keratinocyte differentiation.

The C/EBP members contain a basic leucine zipper (bZIP) domain, which mediates the formation of homodimers or heterodimers with other bZIP family members, at their C-termini (Ramji and Foka, 2002). The dimers bind to the consensus DNA sequences near the core promoters (Akira et al., 1990). Translation of endogenous C/EBPβ mRNA produces three different products: two transactivator isoforms of 46 and 42 kDa (called as the liver-enriched transcriptional activator proteins, LAPs) and one inhibitory isoform of 20 kDa (called as the liver-enriched transcriptional inhibitory protein, LIP) by the use of internal translation initiation sites (Descombes and Schibler, 1991). The LIP consists only of the bZIP domain and acts as a dominant negative form of C/EBPβ (Descombes and Schibler, 1991).

C/EBPβ regulates cellular differentiation and proliferation through the generation of its multiple isoforms. Changes of the relative levels of the isoforms were observed in many cellular processes such as liver development/regeneration (Diehl et al., 1994), mammary grand development (Robinson et al., 1998; Seagroves et al., 1998), and tumorigenic conversion (Raught et al., 1996). The ectopic expression of the LIP isoform in 3T3-L1 cells disrupts terminal differentiation and induces a transformation phenotype (Calkhoven et al., 2000). The LIP isoform expression is also upregulated in mammary epithelial cells by the epidermal growth factor and is associated with cellular proliferation (Baldwin et al., 2004). These findings suggest an important role of the full-length C/EBPβ in inducing cellular differentiation.

A number of studies have demonstrated that C/EBPβ affects the transcription from the HPV promoters in LCR. C/EBPβ binds to a region of the HPV16 LCR and negatively regulates transcription from HPV16 P_{97} (Kyo et al., 1993). The complex of C/EBPβ and YY1 binds to the "switch region" in the HPV18 LCR and activates transcription from the promoter in the LCR (Bauknecht et al., 1996). The downregulation of endogenous C/EBPβ results in enhancement of transcription from the promoter in the HPV11 LCR (Wang et al., 1996).

In this study, we focused on the effect of C/EBPβ on HPV16 P_{97} and P_{670}. HeLa cells and primary human foreskin keratinocytes were transfected with plasmids expressing a reporter (luciferase) gene driven by HPV16 P_{97} or P_{670}, along with a C/EBPβ-expressing plasmid. The overexpression of C/EBPβ in the cells resulted in the activation of P_{670} and repression of P_{97}. The activation of P_{670} was mediated through the direct binding of C/EBPβ to two sites near the promoter.

Results

C/EBPβ was expressed from the plasmid introduced in HeLa cells and human primary foreskin keratinocytes (HFKs)

A newly constructed plasmid for FLAG-tagged C/EBPβ (FLAG-C/EBPβ), pFLAG-C/EBPβ, was introduced into HeLa cells and HFKs to test its expression. The transfection caused no significant morphological changes of the cells within 48 h. Immunoblot analyses of the cell lysates by using anti-C/EBPβ antibody showed that full-length FLAG-C/EBPβ was expressed in the two types of cells, and revealed the presence of two C/EBPβ isoforms, LAP and LIP (Fig. 1A). The endogenous LAP was more abundant than the endogenous LIP in HeLa cells and LIP was not detected in HFKs. In HeLa cells, the expression of FLAG-C/EBPβ induced efficient transcription of the keratin 10 (one of differentiation marker

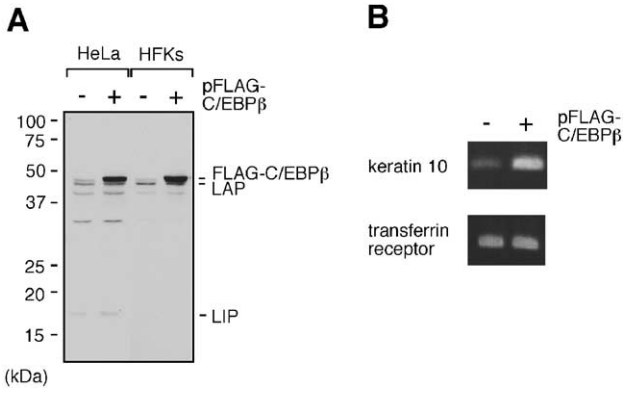

Fig. 1. Expression of FLAG-C/EBPβ in HeLa cells and human foreskin keratinocytes (HFKs). (A) Immunoblot analysis detecting endogenous and exogenous C/EBPβ. HeLa cells or HFKs were transfected with 0.4 ng of pFLAG-C/EBPβ or pFLAG-CMV2. At 48 h after the transfection, total cell extracts were prepared and subjected to immunoblotting with antibodies against the C-terminus of C/EBPβ. FLAG-C/EBPβ: FLAG-tagged full-length C/EBPβ; LAP: endogenous full-length C/EBPβ; LIP: endogenous shorter isoform of C/EBPβ. (B) RT-PCR analysis of mRNA for keratin 10 and transferrin receptor. HeLa cells were transfected with pFLAG-C/EBPβ or pFLAG-CMV2. cDNA libraries were constructed from the mRNAs extracted from the cells at 48 h after the transfection. cDNAs for keratin 10 and transferrin receptor were amplified by PCR with specific primers. The cDNA fragments were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide.

proteins) gene, as reported previously (Zhu et al., 1999), but did not affect transcription of the transferrin receptor gene (Fig. 1B). The results show that exogenous C/EBPβ can be introduced in HeLa cells and HFKs, and at least the one in the former is functional.

Transcription from HPV16 P_{670} was enhanced by C/EBPβ

Two reporter plasmids, pGL3-P_{670} and pGL3-P_{97}, in which a firefly luciferase gene replaced the E1 and E6 genes, were constructed to monitor the transcription from P_{670} and P_{97}, respectively (Fig. 2A). HeLa cells or HFKs were transfected with these reporter plasmids with increasing amounts of pFLAG-C/EBPβ followed by measurement of the luciferase activity. In HeLa cells, increasing FLAG-C/EBPβ enhanced the luciferase activity from pGL3-P_{670} up to 8-fold, but repressed the activity from pGL3-P_{97} down to half (Fig. 2B, left panel). In HFKs, C/EBPβ similarly enhanced and repressed the luciferase activities from pGL3-P_{670} and from pGL3-P_{97}, respectively (Fig. 2B, right panel). The similar effects of C/EBPβ on pGL3-P_{670} and pGL3-P_{97} were observed in HaCat (immortalized human skin keratinocytes) and 293 (a human fibroblast cell line) cells (data not shown). The results show that C/EBPβ enhances and represses transcription from P_{670} and P_{97}, respectively, in these human cells.

The full-length FLAG-C/EBPβ enhanced transcription from P_{670}. C/EBPβ contains a transactivation domain (TAD) at its N-terminus and a basic leucine-zipper (bZIP) type DNA-binding domain at its C-terminus (Fig. 2C, upper panel). To examine the effect of the LIP isoform on transcription from P_{670}, an expression plasmid for the FLAG-C/EBPβ lacking bZIP domain (FLAG-dbZIP) and an expression plasmid for LIP were constructed and used to test for their abilities to enhance transcription from P_{670} in HeLa cells by the transient

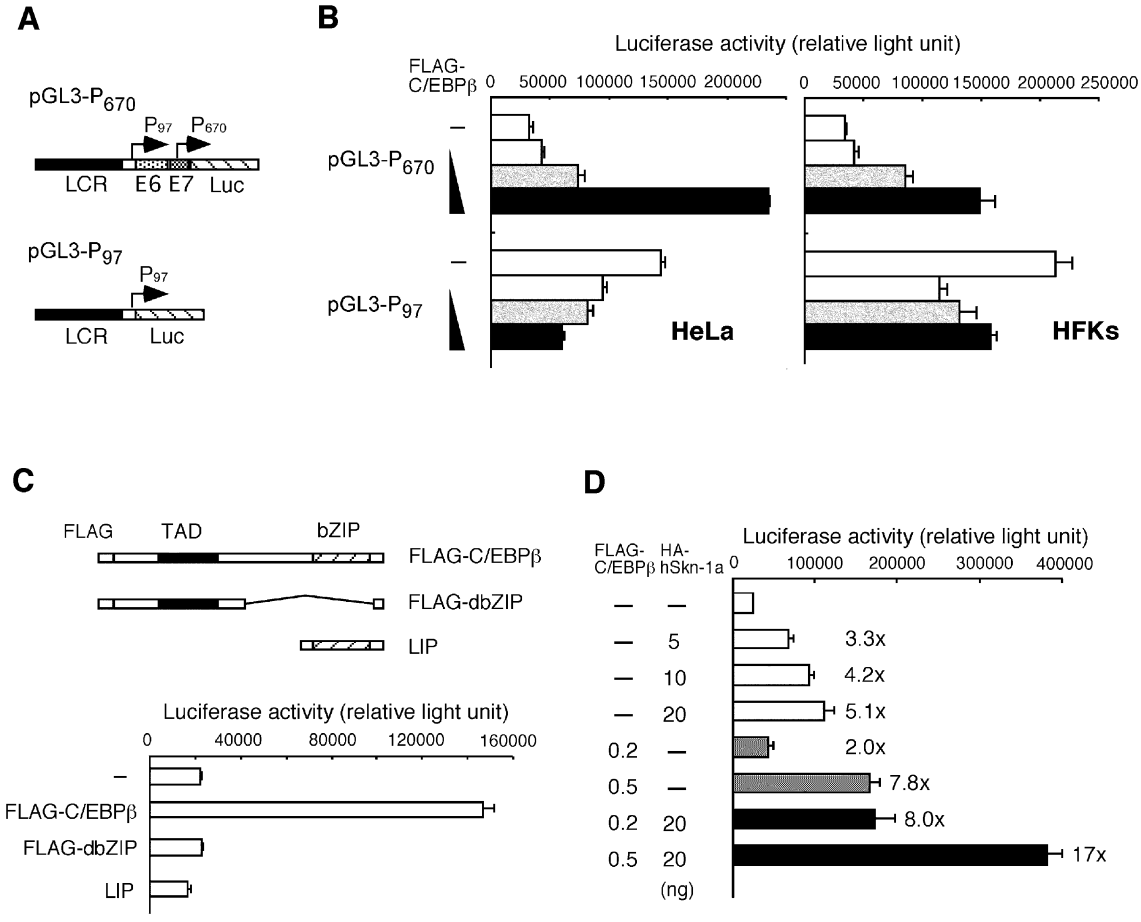

Fig. 2. Activation of HPV16 P_{670} and repression of P_{97} by C/EBPβ. (A) Schematic representation of the HPV16 reporter plasmids, pGL3-P_{670} and pGL3-P_{97}. A firefly luciferase gene was placed in the position of E1 gene (pGL3-P_{670}) or E6 gene (pGL3-P_{97}) to monitor transcription from P_{670} and P_{97}, respectively. (B) Effects of C/EBPβ on expression of luciferase from pGL3-P_{670} and pGL3-P_{97} in HeLa cells and HFKs. Cells were co-transfected with 200 ng of pGL3-P_{670} or pGL3-P_{97} together with increasing amounts of pFLAG-C/EBPβ (0, 0.1, 0.2, and 0.5 ng). At 48 h after the transfection, luciferase activities of cell lysates were measured. Results are presented as means ± standards deviations of three experiments. (C) Effects of domain-deleted C/EBPβs on expression of luciferase from pGL3-P_{670}. HeLa cells were transfected with 200 ng of pGL3-P_{670} and 0.5 ng of pFLAG-CMV2 (backbone plasmid), pFLAG-C/EBPβ, or the expression plasmids for C/EBPβs with deletions (FLAG-dbZIP or LIP). The structures of the C/EBPβs with deletions are schematically illustrated at the top. Luciferase activities of the cell lysates were measured at 48 h after the transfection. (D) Effects of C/EBPβ and hSkn-1a on expression of luciferase from pGL3-P_{670}. HeLa cells were transfected with 200 ng of pGL3-P_{670} and indicated amounts of pFLAG-C/EBPβ and/or pHM/hSkn-1a. The total amounts of the expression plasmids were adjusted to 20.5 ng using pFLAG-CMV2 and pHM6 (backbone plasmid for pHM/hSkn-1a). Activation expressed in multiples was calculated from comparison of the luciferase activities with and without the plasmid expressing hSkn-1a and/or the plasmid expressing C/EBPβ.

reporter assay. FLAG-dbZIP and LIP did not affect transcription from pGL3-P_{670} and only full-length FLAG-C/EBPβ enhanced transcription from pGL3-P_{670} (Fig. 2C, lower panel), indicating that combined function of TAD and bZIP enhanced the transcription from P_{670}.

Enhancing effect by C/EBPβ on the P_{670} transcription was additive to that by the keratinocytes-specific transcription factor hSkn-1a, which also enhances the transcription from P_{670} as shown by our previous study (Kukimoto and Kanda, 2001). HeLa cells were transfected with pFLAG-C/EBPβ, pHM/hSkn-1a (an expression plasmid for HA-tagged hSkn-1a) (Kukimoto and Kanda, 2001), and a mixture of pFLAG-C/EBPβ and pHM/hSkn-1a in the pGL3-P_{670} transient expression assay. Expressions of FLAG-C/EBPβ and HA-hSkn-1a each enhanced luciferase activity, and the combined expression of FLAG-C/EBPβ and HA-hSkn-1a resulted in further enhancement (Fig. 2D). Although the enhancement by HA-hSkn-1a alone reached nearly maximum with 20 ng of pHM/hSkn-1a, co-expression of C/EBPβ induced further enhancement.

RT-PCR detecting hSkn-1a mRNA and Western blotting using anti-hSkn-1a antibody did not show expression of hSkn-1a in HeLa cells transfected with pFLAG-C/EBPβ (data not shown). HA-hSkn-1a also did not increase level of an endogenous C/EBPβ in HeLa cells (data not shown). These results suggest that C/EBPβ and hSkn-1a probably enhance the transcription from P_{670} by independent molecular mechanisms.

The major cis-element(s) required for the C/EBPβ-mediated transcriptional enhancement is located in the E7 region from nt 567 to nt 684

A series of 5′ deletions were introduced into pGL3-P_{670} by digestion with *Nde*I (pGL3-P_{670}/d*Nde*I), *Eco*T22I (pGL3-P_{670}/d*Eco*T22I), or *Pvu*II (pGL3-P_{670}/d*Pvu*II) and expressions of the reporter gene from the deletion mutants were examined in HeLa cells with or without FLAG-C/EBPβ (Fig. 3). Being consistent with the previous data shown by others (Bodily and Meyers, 2005), deletion of total LCR (pGL3-P_{670}/d*Nde*I) resulted in significant reduction of luciferase activity, suggesting that LCR has the enhancer function on P_{670}. However, C/EBPβ-mediated transcriptional enhancement occurred much clearly with the reporters lacking LCR. FLAG-C/EBPβ significantly enhanced luciferase activity from pGL3-P_{670}/d*Nde*I (deletion from the 5′ of LCR to nt 280) up to 16-fold and from pGL3-P_{670}/d*Eco*T22I (deletion from the 5′ of LCR to nt 566) up to 6-fold. The enhancement of transcription did not occur from pGL3-P_{670}/d*Pvu*II (deletion from the 5′ of LCR to nt 684). By a yet unidentified mechanism, luciferase expression from promoter-less plasmids, pGL3-P_{670}/d*Pvu*II and pGL3-Basic, was enhanced by less than 2-fold. The data indicate that the region between nt 567 and nt 684 contains the major element responsible for the enhancement, although there may be minor elements between nt 281 to nt 566.

C/EBPβ bound to the upstream region of P_{670} in vitro

An electrophoretic mobility shift assay (EMSA) demonstrated that a recombinant C/EBPβ protein bound to at least two sites in the HPV16 sequences from nt 501 to nt 670. An LIP isoform fused with glutathione *S*-transferase (GST-LIP) was bacterially expressed and used for the assay, since LIP contains a minimum DNA-binding domain and shows a higher DNA-binding affinity to its target sequence than the full-length C/EBPβ does (Descombes and Schibler, 1991). GST-LIP was incubated with the radiolabeled DNA probes (30 bp) having nucleotide sequences of the HPV16 genome designated A to J (Fig. 4A), and the complex formation of GST-LIP with each probe was detected by mobility shift. GST-LIP clearly bound to probes E and G (Fig. 4B). Faint bindings to the other probes seemed to be non-specific because the bindings were not sequence-specific. A similar binding profile was observed with a recombinant full-length C/EBPβ fused with GST (data not shown).

The probes E and G contain sequences similar to a consensus binding motif for the C/EBP family proteins, $(N)_3$TTGCNNAA$(N)_3$ (Osada et al., 1996) (Fig. 4C). The regions from nt 580 to nt 593 (within probe E) and from nt 601 to nt 614 (within probe G) were designated as CEB#1 and CEB#2, respectively (Fig. 4A). The C/EBP consensus motif partially resembles the binding motif for Skn-1a, WTGCAWNN (W is A or T), and thus the previously described hSkn-1a-binding sites Skn#2 and Skn#3 (Kuki-

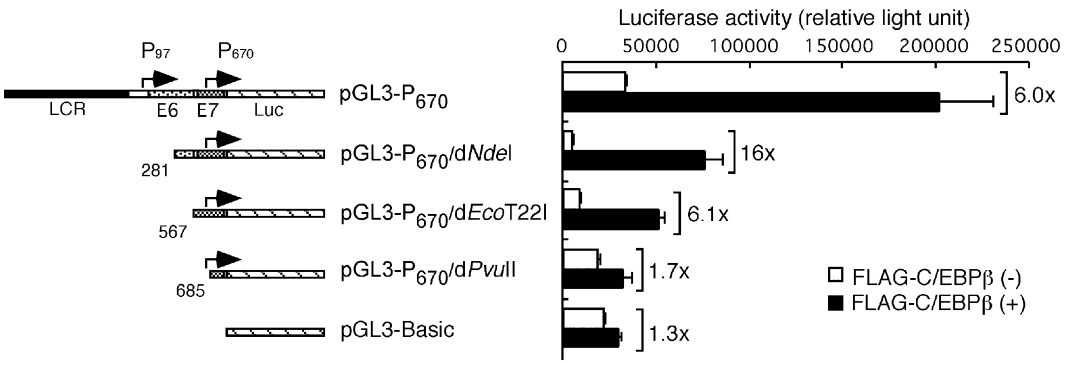

Fig. 3. Effects of C/EBPβ on expression of luciferase from pGL3-P_{670} having 5′-deletion. HeLa cells were transfected with 200 ng of pGL3-P_{670} having 5′-deletion and 0.5 ng of pFLAG-CMV2 or pFLAG-C/EBPβ. The luciferase activities of cell lysates were measured at 48 h after the transfection. Activation expressed in multiples was calculated from comparison of the luciferase activities with and without pFLAG-C/EBPβ for each deleted reporter.

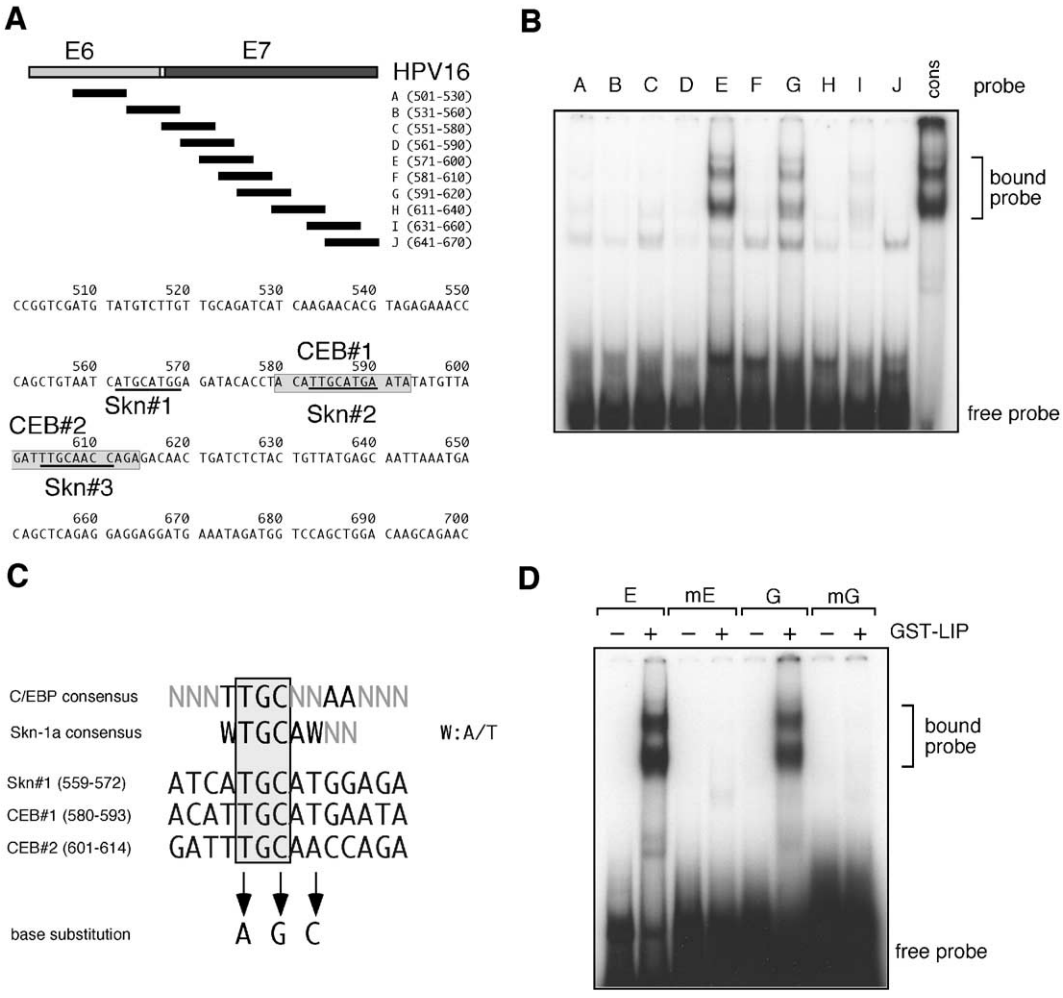

Fig. 4. In vitro binding of C/EBPβ with HPV16 E7 sequences. (A) The DNA probes (A to J) used in electrophoretic mobility shift assay (EMSA). Numbers in parentheses indicate nucleotide numbers of the HPV16 DNA (Los Alamos National Library database). Nucleotide sequence from nt 501 to nt 700 in the HPV16 genome is presented. The previously identified hSkn-1a binding sites are underlined and designated as Skn#1, Skn#2, and Skn#3. Binding sequences for the C/EBP family protein are boxed in gray and designated as CEB#1 and CEB#2. (B) EMSA detecting the complex of GST-LIP with the [^{32}P]-labeled HPV16 probe. The DNA–protein complex was electrophoresed on a 5% polyacrylamide gel and visualized by autoradiography. DNA probe having a consensus C/EBP binding sequence was used as positive control for EMSA (cons). (C) Base substitutions introduced in the probes E and G. (D) Inability of GST-LIP to bind to mutated E and G probes in EMSA. The probes E and G were mutated as shown in panel C to generate probes mE and mG, respectively, and used for EMSA with GST-LIP.

moto and Kanda, 2001) apparently overlap CEB#1 and CEB#2, respectively, on HPV16 DNA (Fig. 4A).

Nucleotide substitutions of TAGG for TTGC (Fig. 4C) were introduced into CEB#1 and CEB#2 in the probes E and G to produce mutated probes mE and mG, respectively. Probes mE and mG totally lost their capabilities of complexing with GST-LIP (Fig. 4D), indicating that the sequences of CEB#1 and CEB#2 are essential for probes E and G, respectively, to bind C/EBPβ.

C/EBPβ bound to the upstream region of P_{670} in vivo

Chromatin immunoprecipitation (ChIP) showed that C/EBPβ bound to the upstream region of P_{670} in HeLa cells. HeLa cells transfected with pGL3-P_{670} together with or without pFLAG-C/EBPβ were cross-linked with formaldehyde and lysed. The lysates were subjected to immunoprecipitation for a DNA–protein complex using either rabbit anti-C/EBPβ antibody or control rabbit IgG. After reversal of cross-links and the proteinase K digestion of the precipitates, a DNA fragment covering HPV16 nt 501 to nt 670 (170 bp) was amplified by PCR and electrophoresed on an agarose-gel (Fig. 5A). The DNA fragment associated with endogenous C/EBPβ was detectable in the precipitate with anti-C/EBPβ antibody and the DNA fragment became prominent in the lysate of HeLa cells transfected with pFLAG-C/EBPβ (Fig. 5A, upper panel). Amplification of Bcl-2 promoter sequence that does not bind to C/EBPβ (Heckman et al., 2003) from the precipitates with anti-C/EBPβ antibody was not successful (Fig. 5A, lower panel), indicating that the amplification is specific to HPV16 P_{670}. Anti-FLAG antibody also precipitated the 170 bp DNA from the lysate of HeLa cells transfected with pFLAG-C/EBPβ (data not shown).

The nucleotide substitutions in CEB#1 and CEB#2 reduced level of DNA precipitated with anti-C/EBPβ antibody. The nucleotide substitutions that abolished bindings of GST-LIP to

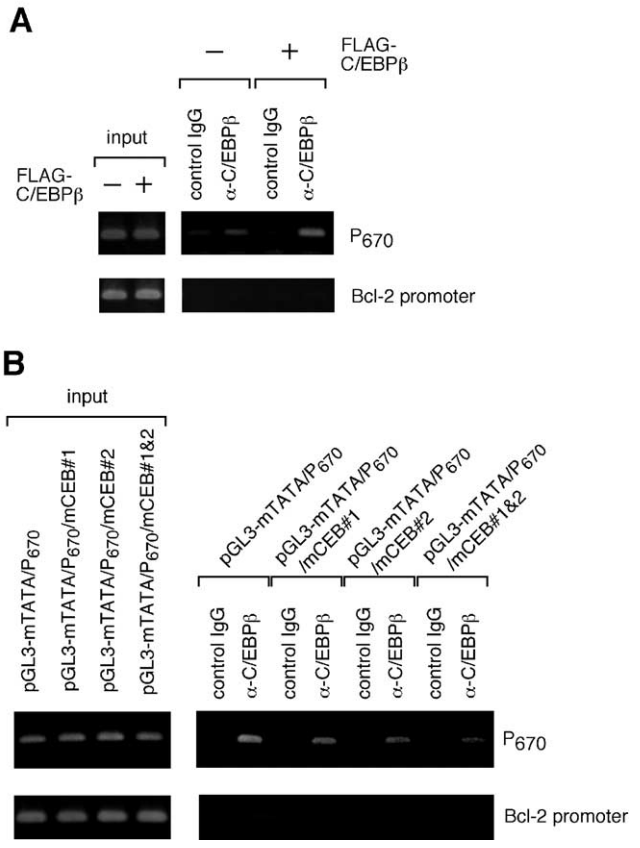

Fig. 5. In vivo binding of C/EBPβ to P_{670} in HeLa cells. (A) Chromatin immunoprecipitation analysis of complex of C/EBPβ and HPV DNA. HeLa cells transfected with pGL3-P_{670} together with pFLAG-CMV2 or pFLAG-C/EBPβ were cultured for 48 h and cross-linked with formaldehyde. The cells were lysed in SDS lysis buffer and sonicated. Chromatin–C/EBPβ complex was immunoprecipitated with anti-C/EBPβ antibody or normal rabbit IgG. DNA was extracted from the precipitate and used as a template for PCR amplification of DNA fragments of nt 501 to nt 670 (P_{670}) and of the Bcl-2 promoter. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Data are representative of three independent experiments. Part (0.2%) of the total input chromatin was used for PCR analyses (input). (B) Chromatin immunoprecipitation analysis of complex of C/EBPβ and HPV DNA with the mutations in C/EBP-binding sites. HeLa cells were transfected with pGL3-mTATA/P_{670} (a TATA box of P_{97} in pGL3-P_{670} was disrupted), pGL3-mTATA/P_{670}/mCEB#1, pGL3-mTATA/P_{670}/mCEB#2, or pGL3-mTATA/P_{670}/mCEB#1&2 together with pFLAG-C/EBPβ, then analyzed by chromatin immunoprecipitation as described in panel A. The nucleotide substitutions introduced into the upstream region of P_{670} in these plasmids are shown in Fig. 4C.

probes E and G (Fig. 4C) were introduced into corresponding regions of pGL3-mTATA/P_{670}, in which a TATA box of P_{97} in pGL3/P_{670} was disrupted to reduce transcription from P_{97}, to produce pGL3-mTATA/P_{670}/mCEB#1 having the mutations in CEB#1, pGL3-mTATA/P_{670}/mCEB#2 having the mutations in CEB#2, and pGL3-mTATA/P_{670}/mCEB#1&2 having the mutations in both CEB#1 and CEB#2. ChIP was conducted with lysates of HeLa cells transfected with pFLAG-C/EBPβ together with one of these plasmids (Fig. 5B). The levels of the 170bp DNA fragments obtained from pGL3-mTATA/P_{670}/mCEB#1 and pGL3-mTATA/P_{670}/mCEB#2 were lower than that from pGL3-mTATA/P_{670}. The level of the 170 bp DNA obtained from pGL3-mTATA/P_{670}/mCEB#1&2 was the lowest. These results strongly suggest that C/EBPβ appears to bind to the upstream region of P_{670} at CEB#1 and CEB#2 in a sequence-specific manner in vivo.

The nucleotide substitutions in CEB#1 and CEB#2 abolished the C/EBPβ-mediated enhancement of transcription from P_{670}

The transcription from the reporter lacking capability of binding with C/EBPβ was examined by introducing the nucleotide substitutions into CEB#1 and CEB#2. To know the possible contribution of P_{97} to the transcription from pGL3-P_{670}, luciferase activity from pGL3-P_{670} was compared with that from pGL3-mTATA/P_{670} in which TATA-box of P_{97} in pGL3-P_{670} was abolished by the nucleotide substitutions as previously described (Kukimoto and Kanda, 2001). The luciferase activity from pGL3-P_{670} was slightly higher than that from pGL3-mTATA/P_{670} (Fig. 6, upper panel), indicating that P_{97} contributes to the transcription from P_{670} in pGL3-P_{670}. The P_{97} contribution was clearer in HFKs than in HeLa cells (Fig. 6,

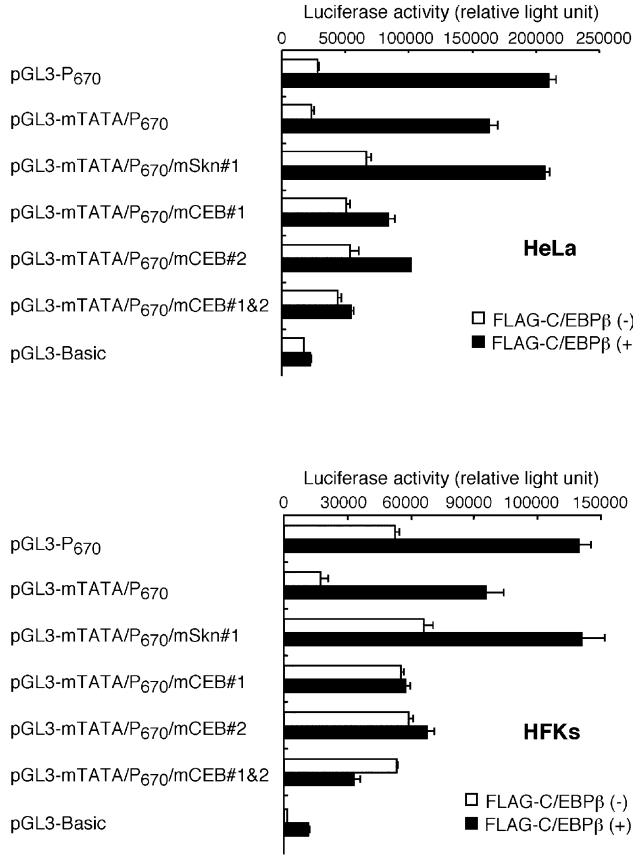

Fig. 6. Effects of C/EBPβ on expression of luciferase from pGL3-P_{670} with the mutations in C/EBP-binding sites in HeLa cells and HFKs. HeLa cells or HFKs were transfected with 200 ng of pGL3-mTATA/P_{670} (a TATA box of P_{97} in pGL3-P_{670} was disrupted), pGL3-mTATA/P_{670}/mSkn#1, pGL3-mTATA/P_{670}/mCEB#1, pGL3-mTATA/P_{670}/mCEB#2, or pGL3-mTATA/P_{670}/mCEB#1&2 together with 0.5 ng of pFLAG-CMV2 (bars in gray) or pFLAG-C/EBPβ (bars in black). The nucleotide substitutions introduced into the upstream region of P_{670} in these plasmids are shown in Fig. 4C. At 48 h after the transfection, luciferase activities of the cellular extracts were measured. Results are presented as means ± standards deviations of three experiments. Upper and lower panels show data obtained by using HeLa cells and HFKs, respectively.

lower panel). Therefore, for further mutational analyses of CEB#1 and CEB#2, pGL3-mTATA/P_{670} was used as a parental plasmid. Luciferase expressions in HeLa cells transfected with pGL3-mTATA/P_{670}/mCEB#1 and pGL3-mTATA/P_{670}/mCEB#2 were not efficiently enhanced by expression of FLAG-C/EBPβ (Fig. 6, upper panel). Luciferase expression from pGL3-mTATA/P_{670}/mCEB#1&2 was not enhanced. Nucleotide substitutions introduced into hSkn-1a-binding site #1 (pGL3-mTATA/P_{670}/mSkn#1) did not affect C/EBPβ-mediated enhancement. Similar results were obtained in HFKs (Fig. 6, lower panel). The results strongly support the idea that the direct binding of C/EBPβ to CEB#1 and CEB#2 is required for the enhancement of transcription from P_{670} by C/EBPβ.

Discussion

Activation of the HPV late promoter occurs in host cells that have started terminal differentiation (Longworth and Laimins, 2004). Therefore, it is reasonable to speculate that the late promoter is activated by some of the cellular transcription factors regulating cell differentiation. In this study, we examined the involvement of C/EBPβ, a key transcription factor that induces the terminal differentiation of keratinocytes (Zhu et al., 1999), in the regulation of the HPV16 promoters. It was found that C/EBPβ bound to two sites in the upstream region of the P_{670} late promoter in a sequence-specific manner and enhanced the transcription in HeLa cells and HFKs. As reported previously (Kyo et al., 1993), C/EBPβ repressed the transcription from the P_{97} early promoter.

C/EBPβ is known to interact with SWI/SNF chromatin remodeling complexes and induces transcription of several cellular genes in a chromatin environment (Kowenz-Leutz and Leutz, 1999). A recent study of an episomal HPV31 genome maintained in a human cervical cell line showed that a nucleosome-free region appears in the upstream region of the late promoter upon host cell differentiation (del Mar Pena and Laimins, 2001). The appearance of the nucleosome-free region strongly coincides with induction of the transcription from the late promoter. It is possible that C/EBPβ-mediated recruitment of the SWI/SNF chromatin remodeling complex to the upstream region of the HPV late promoter induces rearrangement of the nucleosome structure around the promoter, resulting in the appearance of a nucleosome-free region where transcription takes place.

The sequences of CEB#1 and CEB#2 are present in the corresponding positions in the E7 sequences of HPV31 and HPV18, strongly suggesting that the late promoters of HPV31 and HPV18 are activated by C/EBPβ. The identical sequences are not present in the E7 sequences of HPV6, HPV11, HPV33, and HPV58. However, substantial variations are tolerated for C/EBP-binding sequences (Osada et al., 1996), and several sequences containing TTGC are present in the E7 region of these HPVs. It remains to be examined experimentally whether C/EBPβ enhances transcription from the late promoter of these HPVs through its direct binding to the upstream region.

We have previously shown that hSkn-1a, a POU-transcription factor inducing keratinocyte differentiation, binds to Skn#1 and Skn#2 (Fig. 4A) and activates HPV16 P_{670} by displacement of YY1 repressor (Kukimoto and Kanda, 2001). hSkn-1a does not bind to the region previously designated as Skn#3. When both C/EBPβ and hSkn-1a were expressed in HeLa cells, the enhancement of transcription from P_{670} seemed to be additive (Fig. 2D). Probably hSkn-1a binds to Skn#1 and C/EBPβ binds to CEB#2 (Fig. 4A). Since CEB#1 and Skn#2 partially overlap (Figs. 4A and C), it is not clear which of C/EBPβ and hSkn-1a binds to the site dominantly. Our real-time RT-PCR analysis of differentiating primary keratinocytes showed that induction of hSkn-1a preceded induction of C/EBPβ during differentiation (T. Takeuchi et al., unpublished data). It is therefore possible that hSkn-1a first binds to Skn#2 and relieves the YY1-mediated repression, and then hSkn-1a is displaced by C/EBPβ.

C/EBPβ repressed transcription from HPV16 P_{97} as described previously (Kyo et al., 1993). C/EBPβ disrupts the binding of TATA-binding protein to the TATA box in the early promoter of HPV18 (Bauknecht and Shi, 1998). It is possible that the reduction of E6 and E7, which are produced from transcripts from P_{97} and have function to delay the terminal differentiation, is required for the later stage of the differentiation-associated viral propagation.

This study has shown that C/EBPβ enhances the transcription from the HPV16 late promoter. In the epidermis, C/EBPβ is expressed at a low level in the basal cell layers where the HPV late genes are not transcribed and at a high level in the middle and upper stratum spinosum where the HPV late genes are actively transcribed. It is very likely that C/EBPβ is involved in the differentiation-associated life cycle of HPV16.

Materials and methods

Construction of plasmids

The cDNA of C/EBPβ was provided by DNA Bank, BioRecource Center, RIKEN (Ibaraki, Japan), and was inserted in flame between *Eco*RI and *Sal*I sites of pFLAG-CMV2 (Sigma-Aldrich, St. Louis, MO) to generate an expression plasmid for an N-terminal FLAG-tagged C/EBPβ, pFLAG-C/EBPβ. The expression plasmid for C/EBPβ with deletion of the DNA-binding domain was constructed by digestion of pFLAG-C/EBPβ with *Sac*I and self-ligation. The DNA fragment encoding an LIP isoform of C/EBPβ was synthesized by PCR using pFLAG-C/EBPβ as a template with primers (forward, 5′-CGA ATT CGC CAT GGC GGC GGG CTT CCC GTA-3′; reverse, 5′-GCG TCG ACT CTA GCA GTG GCC GGA-3′; restriction digestion sites were underlined), and was inserted into pCMV5 (a kind gift from Dr. Michael Megner) as a *Eco*RI–*Sal*I fragment to generate an expression plasmid for LIP. The bacterial expression plasmid for an LIP fused with glutathione *S*-transferase (GST-LIP) was made by insertion of the *Eco*RI–*Sal*I fragment of LIP into pGEX-6P-1 (Amersham Biosciences, Piscataway, NJ).

To construct the P_{670} reporter plasmid, a *Pst*I–*Nco*I fragment of HPV16 (from nt 7003 to 7904 and from nt 1 to 868) was inserted between *Sma*I and *Nco*I sites of pGL3-Basic

(Promega, Madison, WI). In the resultant plasmid pGL3-P_{670}, the HPV16 DNA was fused with the luciferase DNA at the first ATG codon of the HPV16 E1 gene. To construct the P_{97} reporter plasmid pGL3-P_{97}, a DNA fragment of HPV16 (from nt 7003 to 7904 and from nt 1 to 101) was synthesized by PCR using primers (forward, 5′-CCC AAG CTT CTG CAG ACC TAG ATC AGT-3′; reverse, 5′-CAT GCC ATG GCA GTT CTC TTT TGG TGC ATA A-3′; restriction digestion sites were underlined), and was inserted between *Hin*dIII and *Nco*I sites of pGL3-Basic. Deleted P_{670} reporter plasmids, pGL3-P_{670}/d*Nde*I, pGL3-P_{670}/d*Eco*T22I, and pGL3-P_{670}/d*Pvu*II, were made by digestion of pGL3-P_{670} with *Sac*I and *Nde*I, with *Sac*I and *Eco*T22I, and with *Sac*I and *Pvu*II, respectively, followed by blunting and self-ligation. All nucleotide substitutions in the reporter constructs were introduced using a Mutan-Super Express Km site-directed mutagenesis kit (Takara, Osaka, Japan) or standard PCR techniques based on KOD-plus polymerase (TOYOBO, Osaka, Japan). All mutations were verified by sequencing.

Cell culture and luciferase assay

HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. Primary human foreskin keratinocytes (HFKs) were purchased from Kurabo (Osaka, Japan), and grown in serum-free keratinocyte growth medium (Kurabo) in 5% CO_2 at 37 °C. For luciferase assays, 3×10^4 HeLa cells or 4×10^4 HFKs were seeded onto 24-well plates. HeLa cells and HFKs were cultured for 4 h and overnight, respectively, before transfection. The cells were transfected with 200 ng of luciferase reporter plasmids and 0.1–0.5 ng of pFLAG-C/EBPβ by using FuGENE-6 reagent (Roche Applied Science, Indianapolis, IN). To monitor transfection efficiency, the cells were co-transfected with 5 ng of the cytomegalovirus promoter-driven *Renilla*-luciferase plasmid. Firefly and *Renilla* luciferase activities were measured at 48 h after transfection by using the Dual-Glo luciferase assay kit (Promega) and a TopCount microplate luminometer (Perkin-Elmer Sciences Inc, Boston, MA). Firefly luciferase activities were normalized using *Renilla*-luciferase activities. Each experiment was done in triplicate and repeated at least three times.

Western blot analysis

Total extracts of 1.2×10^5 HeLa cells or 1.6×10^5 HFKs transfected with 0.4 ng of pFLAG-C/EBPβ or pFLAG-CMV2 were electrophoresed on a 12% SDS-Polyacrylamide gel. After transfer of proteins to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), the membrane was blocked with 5% skim milk in PBS-0.1% Tween 20 at room temperature and then incubated with rabbit anti-C/EBPβ antibody (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The membranes were washed and incubated with peroxidase-conjugated goat anti-rabbit antibody for 1 h. Peroxidase activity was detected with the enhanced chemiluminescence detection method (ECL plus kit, Amersham Biosciences).

Electrophoretic mobility shift assay

The GST-LIP fusion protein was expressed in *Escherichia coli* strain JM109 and purified by GSTrap affinity-column chromatography (Amersham Biosciences) with AKTA*prime* (Amersham Biosciences). A mixture of double-stranded [^{32}P]-labeled oligonucleotides (0.4 pmol), 1 μg of GST-LIP, and 1 μg of poly (dI/dC) in a final volume of 10 μl of binding buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 40 μg/ml BSA) was incubated at room temperature for 30 min. Then, the samples were loaded on a 5% polyacrylamide gel and electrophoresed in 0.5× Tris–borate/EDTA buffer at room temperature. The gels were dried and visualized by autoradiography on X-ray films. The sense sequences of double-stranded oligonucleotides are as follows: A (501–530), 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′; B (531–560), 5′-CAA GAA CAC GTA GAG AAA CCC AGC TGT AAT-3′; C (551–580), 5′-CAG CTG TAA TCA TGC ATG GAG ATA CAC CTA-3′; D (561–590), 5′-CAT GCA TGG AGA TAC ACC TAC ATT GCA TGA-3′; E (571–600), 5′-GAT ACA CCT ACA TTG CAT GAA TAT ATG TTA-3′; F (581–610), 5′-CAT TGC ATG AAT ATA TGT TAG ATT TGC AAC-3′; G (591–620), 5′-ATA TAT GTT AGA TTT GCA ACC AGA GAC AAC-3′; H (611–640), 5′-CAG AGA CAA CTG ATC TCT ACT GTT ATG AGC-3′; I (631–660), 5′-TGT TAT GAG CAA TTA AAT GAC AGC TCA GAG; J (641–670), 5′-AAT TAA ATG ACA GCT CAG AGG AGG AGG ATG-3′; mE, 5′-GAT ACA CCT ACA TAG GAC GAA TAT ATG TTA-3′; mG, 5′-ATA TAT GTT AGA TTA GGA CCC AGA GAC AAC-3′; C/EBP-cons, 5′-AAG CTG CAG ATT GCG CAA TCT GCA GCT T-3′. Numbers in parentheses indicate nucleotide numbers of the HPV16 genome (the HPV Sequence Database of Los Alamos National Laboratory) and nucleotides used for substitution mutations are underlined.

Chromatin immunoprecipitation

HeLa cells (8×10^5 cells) were grown on a 100-mm dish for 4 h and then transfected with 6 μg of the reporter construct and 6 ng of pFLAG-C/EBPβ or pFLAG-CMV2 using FuGENE-6. At 48 h after the transfection, the cells were cross-linked with 1% formaldehyde for 5 min at 37 °C followed by a 5-min treatment with 125 mM glycine to quench cross-linking. The cross-linked cells were lysed in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.0) supplemented with protease inhibitor mixture (Roche Applied Science), incubated on ice for 10 min, and sonicated using a Bioruptor (Cosmobio, Tokyo, Japan). The sonication resulted in an average DNA fragment size of 500 bp. One hundred microliters of the sonicated materials was diluted with 900 μl of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris–HCl, pH 8.0) and precleared with salmon sperm DNA/protein G-agarose beads. Part (2%) of the precleared lysate was taken as input chromatin for PCR analyses. The supernatant was incubated overnight at 4 °C with anti-C/EBPβ (Santa Cruz) or normal rabbit IgG. The chromatin–antibody complex was collected through incuba-

tion with the agarose beads for 2 h at 4 °C and centrifugation. The beads were washed sequentially for 5 min at 4 °C in wash buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, Tris–HCl, pH 8.0), wash buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, Tris–HCl, pH 8.0), wash buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0), and TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) twice, then extracted with 200 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$, 10 mM DTT). The eluted chromatin and the input chromatin were heated at 65 °C for 4 h to reverse the cross-links, followed by proteinase K digestion overnight at 37 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The purified DNA fragments were used as templates for PCR amplification to detect the protein occupancy of the HPV P_{670}. PCR primers were as follows: forward, 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′; reverse, 5′-CAT CCT CCT CCT CTG AGC TGT CAT TTA ATT-3′. These primers amplify 170 bp DNA from nt 501 to nt 670 of the HPV16 sequence. PCR consisted of one cycle of initial denaturation at 94 °C for 1 min and 21 cycles of the following conditions: denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. Human Bcl-2 promoter sequence (from −748 to −587) (Heckman et al., 2003) was amplified as a negative control for ChIP analysis. PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide.

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